Effects of Inhibiting Antioxidant Pathways on Cellular Hydrogen Sulfide and Polysulfide Metabolism

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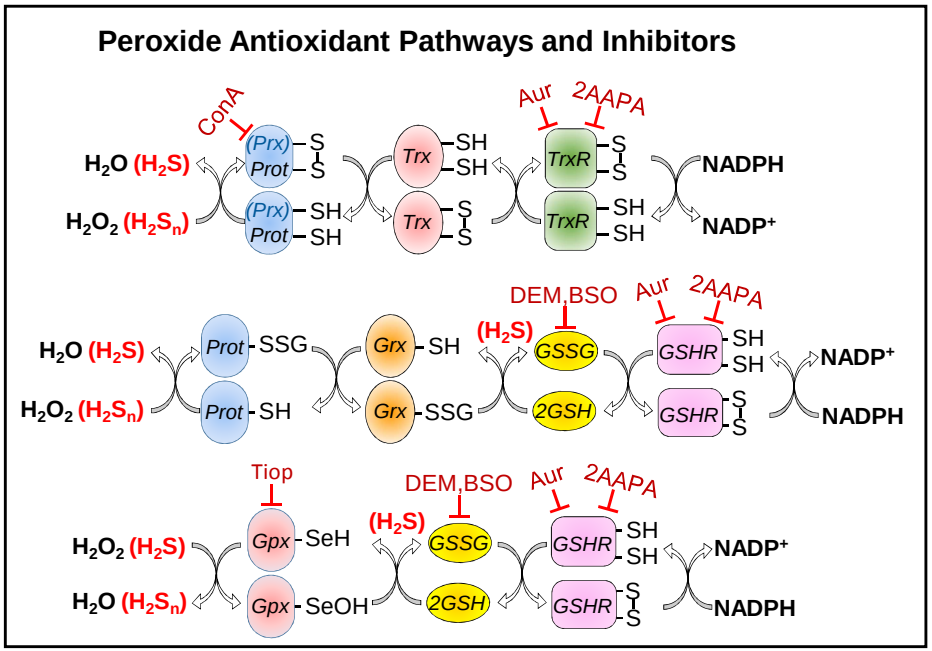
ABSTRACT

Elaborate antioxidant pathways have evolved to minimize the threat of excessive reactive oxygen species (ROS) and to regulate ROS as signaling entities. ROS are chemically and functionally similar to reactive sulfide species (RSS) and both ROS and RSS have been shown to be metabolized by the antioxidant enzymes, superoxide dismutase and catalase. Here we use fluorophores to examine the effects of a variety of inhibitors of antioxidant pathways on metabolism of two important RSS, hydrogen sulfide (H_2S with AzMC) and polysulfides (H_2S_n, where n=2-7, with SSP4) in HEK293 cells. Cells were exposed to inhibitors for up to 5 days in normoxia (21% O_2) and hypoxia (5% O_2), conditions also known to affect ROS production. Decreasing intracellular glutathione (GSH) with L-buthionine-sulfoximine (BSO) or diethyl maleate (DEM) decreased H_2S production for 5 days but did not affect H_2S_n. The glutathione reductase inhibitor, auranofin, initially decreased H_2S and H_2S_n but after two days H_2S_n increased over controls. Inhibition of peroxiredoxins with conoidin A decreased H_2S and increased H_2S_n, whereas the glutathione peroxidase inhibitor, tiopronin, increased H_2S. Aminoadipic acid, an inhibitor of cystine uptake did not affect either H_2S or H_2S_n. In buffer, the glutathione reductase and thioredoxin reductase inhibitor, 2-AAPA, the glutathione peroxidase mimetic, ebselen, and tiopronin variously reacted directly with AzMC and SSP4, reacted with H_2S and H_2S_2, or optically interfered with AzMC or SSP4 fluorescence. Collectively these results show that antioxidant inhibitors, generally known for their ability to increase cellular ROS, have various effects on cellular RSS. These findings suggest that the inhibitors may affect cellular sulfur metabolism pathways that are not related to ROS production and in some instances they may directly affect RSS or the methods used to measure them. They also

illustrate the importance of carefully evaluating RSS metabolism when biologically or pharmacologically attempting to manipulate ROS.

Key Words: reactive sulfide species; reactive oxygen species; antioxidants


Peroxide Antioxidant Pathways and Inhibitors
ConA
Aur
2AAPA
H2O (H2S)
H2O2 (H2S2)
(Prx)
Prot
Trx
TrxR
NADPH
NADP+
DEM,BSO
Prot
SSG
SH
Grx
GSSG
2GSH
GSHR
Tiop
Gpx
SeH
SeOH

1. Introduction

It is becoming more apparent that redox environments in various intracellular compartments are physiologically regulated to optimize electron transfer and prevent electrons from 'wandering off' and disrupting cellular processes. Although, numerous oxidants can arise from a variety of intracellular processes, or be ingested in the diet, the production of reactive oxygen species (ROS), namely peroxide (H_2O_2) has received the most attention as it has been implicated a variety of aspects of oxidative stresses from signaling (eustress) to mobilization of antioxidant responses (oxidative stress), and if this fails the resulting oxidative distress can lead to catastrophic consequences [1-19].

Regulation of, and protection from ROS is attributed to a hierarchy of antioxidant mechanisms that include ROS buffers and a compliment of enzymes that facilitate transfer of electrons from NADPH to the oxidant or oxidized cellular constituent, the latter often a thiol on a regulatory or structural protein. Thiols are also the redox currency of the major intracellular redox buffer, glutathione, and the cadre of redox enzymes, peroxiredoxins, thioredoxins, thioredoxin reductases, glutaredoxins and glutathione reductases employed to maintain this balance. Chemically targeting these antioxidant systems has been used to examine their relative regulatory roles as well as to disrupt redox balance as a therapeutic tool [20-31].

We have noted striking similarities between ROS and reactive sulfide species (RSS) and have proposed that in some instances the former could be mistaken for the latter in cellular functions [32]. Sequential one-electron reductions of O_2 produces superoxide($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and H_2O, whereas one-electron oxidation of hydrogen sulfide (H_2S) progressively forms a thiyl radical ($HS^{\bullet}$), hydrogen persulfide (H_2S_2) and persulfide "supersulfide" radical ($HS_2^{\bullet-}$) before terminating in elemental sulfur (S_2), the latter usually

cyclizing to S_8. The primary target of both peroxide and persulfide is the above-mentioned cysteine on regulatory proteins and the effects of peroxidation or persulfidation on the function of a number of proteins has been shown to be identical [17,33-40]. Furthermore, many methods routinely employed to measure ROS also detect RSS, at times with greater sensitivity [41].

Although the "Redox Code" posits that concept of antioxidant protection was believed to be "richly elaborated in an oxygen-dependent life, where activation/deactivation cycles involving O_2 and H_2O_2 contribute to spatiotemporal organization for differentiation, development, and adaptation to the environment" [42], an argument can be made for antioxidants in the regulation of RSS. Essentially all antioxidant buffers and above-mentioned enzymes appeared within the first several hundred million years after life's beginnings 3.8 billion years ago [43-51]. At this time the oceans were anoxic and sulfidic [52,53] and it would take over a billion years for the "great oxidation event" to slightly nudge atmospheric O_2. It wasn't until 600 million years ago, after nearly seven-eighths of evolution had passed, before the oceans became oxic. As the origins of antioxidant mechanisms likely coincided with the advent of anoxygenic photosynthesis, a process that has been proposed to oxidize H_2S to polysulfides and reduce CO_2 to methane and other organic compounds [32] it would seem that control of sulfur-based redox reactions would have been a priority, perhaps not only for redox balance but for general metabolism. We proposed that many of these pathways remain in extant animals and perform similar functions.

We recently examined the possibility that sulfur was metabolized by two antioxidant enzymes, superoxide dismutase (SOD) and catalase, and indeed this appeared to be the case. Both Cu/Zn and Mn SOD oxygen-dependently oxidized H_2S to polysulfides, initially forming persulfide (H_2S_2) and subsequently longer chain polysulfides (H_2S_n where n=3-5) but did not

metabolize polysulfides [54]. Bovine catalase also oxidized H_2S to polysulfides in the presence of either O_2 or H_2O_2, whereas in hypoxia it produced H_2S from either thioredoxin or the sulfur-reductant dithiothreitol (DTT), a process that required NADPH [55]. Furthermore, the partial pressure of oxygen at which the catalase switched from an oxidase to a reductase (P_{50}) which is 20 mmHg, is striking similar to the P_{50} of the oxyhemoglobin saturation curve (26 mmHg) suggesting that catalase, which is abundant in red blood cells, oxidizes H_2S during normoxia, whereas as blood Po_2 falls, not only is O_2 unloaded from hemoglobin, but vasodilator H_2S is produced to augment O_2 delivery.

In the present study we further examine sulfur (RSS) metabolism in HEK293 cells by blocking endogenous antioxidant pathways while monitoring endogenous H_2S and polysulfides with specific fluorophores (AzMC and SSP4, respectively). We show that these antioxidant inhibitors have profound effects on cellular RSS that are not consistent with concomitant effects on ROS and propose that some of the effects of manipulating cellular antioxidant pathways may be mediated by RSS.

2. Materials and methods

2.1. Chemicals

SSP4 (3', 6'-Di(O-thiosalicyl)fluorescein) was purchased from Dojindo molecular Technologies Inc. (Rockville, MD). All other chemicals were purchased either from Sigma-Aldrich (St. Louis, MO) or ThermoFisher Scientific (Grand Island, NY). Please note that we use H_2S to denote the total sulfide added (sum of H_2S + HS^-) usually derived from Na_2S. Also, while S^{2-} is often thought as part of the H_2S + HS^- equilibrium, it does not exist under

97 physiological conditions [56]. Phosphate buffer (PBS; in mM): 137 NaCl, 2.7, KCl, 8 Na_2HPO_4,
98 2 NaH_2PO_4. pH was adjusted with 10 mM HCl or NaOH to 7.4.
99
100 *2.2. Effects of inhibitors on sulfur metabolism in cells*
101 Human embryonic kidney (HEK293) cells were cultured and maintained at 37^o C in a 5%
102 CO_2 humidified incubator with 21% O_2 supplemented with DMEM (low glucose) containing
103 10% FBS and 1% Pen/Strep. In a typical experiment they were transferred from a T-25 tissue
104 culture flask to two 96 well plates with gas-permeable bottoms (Coy Laboratory Products, Inc.
105 grass Lake, MI) and grown to 80–95% confluency. The cells were then treated with either an
106 H_2S sensitive fluorophore 7-azido-3-methylcoumarin (AzMC, 25 μM) or a polysulfide sensitive
107 fluorophore 3',6'-Di(O-thiosalicyl)fluorescein (SSP4, 10 μM). Excitation/emission wavelengths
108 for AzMC and SSP4 were 365/450 and 482/515, respectively per manufacture's
109 recommendations. Fluorescence was measured on a SpectraMax M5e plate reader (Molecular
110 Devices, Sunnyvale, CA) according to the manufacturer's recommendations Inhibitors of H_2S
111 biosynthesis or sulfur doors were typically added after an initial baseline reading and one plate
112 was returned to the 21% O_2 incubator (normoxia) and the other placed in a model 856-HYPO
113 hypoxia chamber (Plas Labs, Inc. Lansing, MI) and incubated in 5% O_2/5% CO_2 balance N_2
114 (hypoxia) at 37^o C for 3-5 days. Previous studies have shown that this prolonged hypoxia
115 produces a sustained increase in cellular H_2S and these studies were designed to determine if any
116 inhibitors specifically affected the hypoxic response.
117
118 *2.3. Effects of inhibitors on fluorescence and sulfur metabolism in buffer*

The inhibitors used in this study could give an erroneous impression of an effect on cellular sulfur metabolism if they directly interacted with AzMc and SSP4 or H_2S and polysulfides. Three groups of experiments were performed to examine these possibilities. In the first group, the inhibitors were added directly to the fluorophores to determine if they specifically activated the fluorophores. In the second group, the inhibitors were added to concomitantly with 200 μM H_2S and either AzMC or SSP4. A decrease in AzMC fluorescence would suggest that either the inhibitors catalyzed H_2S consumption or there was a direct interference of the reaction between H_2S and AzMC. An increase in SSP4 fluorescence in these experiments would suggest that the inhibitors catalyzed the oxidation of H_2S to polysulfides. In the third group of experiments 10 μM of H_2S was incubated with AzMC, or 30 μM of K_2S_n was incubated with SSP4 for 120 min to determine if the inhibitors interfered with fluorescence of AzMC or SSP4 after thethe fluorophores were activated by the sulfur moiety. In these experiments the H_2S and K_2S_n concentrations were chosen to produce approximately the same fluoresence as that observed in the cell experiments and 120 min allowed for the fluorophore-sulfur reaction to be completed.

The compounds of interest were aliquoted into black 96 well plates in a darkened room and fluorescence was measured on a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA). Fluorescence was typically measured every 10 min over 90 min. The inhibitor concentrations were selected to bracket the concentrations used in the cell experiments. All experiments were performed at room temperature (20^o C).

2.4. Cell viability

Cell viability was determined using the PrestoBlue fluorophore per manufacturer's directions. PrestoBlue was either added at the end of an experiment or to PBS and fluorescence measured 1 and 2 h later at 535/615 nm (Ex/Em).

2.5. Verification of AzMC specificity

An anonymous reviewer expressed some concern regarding the specificity of AzMC as a H_2S fluorophore. To examine this we first examined the effects of GSH, -3MP, H_2O_2 and the NO donor sodium nitroprusside on AzMC fluorescence in PBS. We then incubated HEK293 cells with inhibitors of H_2S biosynthesis, propargylglycine (PPG; 10 mM) and 1 mM aminooxyacetate (AOA; 1 mM). PPG is a relatively selective inhibitor of cystathionine γ lyase and AOA is an inhibitor of both CSE and cystathionine β-synthase (CBS).

2.6. Calculations

Results are expressed as mean +SE. Statistical analysis was determined by one-way ANOVA with Holm-Sidak for multiple comparisons. Significance was assumed at $p \leq 0.05$.

3. Results

3.1. Effects of BSO and DEM on intracellular H_2S and polysulfides in HEK293 cells

Both L-buthionine-sulfoximine (BSO) and diethyl maleate (DEM) decreased intracellular GSH and increased ROS in primary cortical cultures containing both neurons and astrocytes [57]. The concentration-dependent effects of BSO and DEM on H_2S (AzMC fluorescence) in both normoxic (21% O_2) and hypoxic (5% O_2) HEK293 cells are shown in **Fig. 1**. Although the BSO-hypoxia control cells were lost due to medium leaking from the wells, there was a clear

decrease in AzMC fluorescence between 0.3 μM BSO and 1 μM BSO supporting the conclusion that both BSO and DEM concentration-dependently decreased AzMC fluorescence in normoxic and hypoxic cells. The effects of BSO and DEM on polysulfides (SSP4 fluorescence) in HEK293 cells in hypoxia (5% O_2) are also shown in **Fig. 1**. Neither BSO nor DEM greatly affected SSP4 fluorescence, the rapid initial increase in SSP4 fluorescence in all samples likely reflects a large pre-existing polysulfide pool in these cells and may have masked specific effects of the inhibitors.

In order to determine if the effects of BSO or DEM were on cellular H_2S and polysulfides we then explored potential interactions of the inhibitors with fluorophores in buffer (**Figs. S1, S2**, respectively). Neither BSO nor DEM directly reacted with otherwise unactivated fluorophore, nor did they affect the reaction of the fluorophores with H_2S or with H_2S-activated AzMC or K_2S_n-activated SSP4. These results suggest that both BSO and DEM directly inhibit intracellular H_2S production but do not affect endogenous polysulfides or polysulfide production.

3.2. Effects of cystine, BSO and DEM on H_2S and polysulfide metabolism in HEK293 cells

Although both BSO and DEM decrease intracellular GSH and increase ROS, DEM increases cystine uptake while BSO decreases it [57]. In order to determine if cystine affected the actions of these inhibitors on cellular sulfur metabolism we incubated HEK293 cells with combinations of 200 μM cystine, 10 μM BSO and 100 μM DEM (**Fig. 2**). Cystine decreased intracellular H_2S, but it was less than half as effective as either BSO or DEM and the effects of either BSO or DEM were not affected by addition of cystine. SSP4 fluorescence was unaffected by cysteine, BSO, DEM, or by combinations of cystine and BSO or cystine and DEM. These

results suggest that the effects of BSO and DEM on intracellular H_2S metabolism are independent of cystine uptake and not related to their effects on intracellular ROS.

Figure 2 also shows that cystine alone decreases AzMC but does not affect SSP4 fluorescence in HEK293 cells. Cystine also had direct effects on the fluorophores in buffer but these were opposite of those produced in cells (**Fig. S3**). Cystine slightly increased fluorescence of both AzMC and SSP4 alone and it concentration-dependently increased SSP4 fluorescence when added in conjunction with H_2S. Cystine produced approximately a 20% increase in AzMC fluorescence when added 120 min after AzMC but this was not dependent on cysteine concentration; cystine did not affect fluorescence of SSP4 pre-incubated with K_2S_n (**Fig. S4**). The effects of cystine on cellular polysulfides remain to be clarified.

3.3. Effects of auranofin on intracellular H_2S and polysulfides in HEK293 cells

Auranofin is an irreversible inhibitor of glutathione reductase (GSHR) and increases intracellular ROS [27,28,58,59]. The concentration-dependent effects of auranofin on sulfur metabolism are shown in **Fig. 3**. Auranofin concentration-dependently decreased the time dependent rate of both AzMC and SSP4 fluorescence increase in normoxic and hypoxic cells.

The effects of auranofin in buffer are shown in **Fig. S5**. In buffer, auranofin did not directly react with otherwise unactivated fluorophore nor did it affect the reaction of AzMC with H_2S. However, auranofin decreased SSP4 fluorescence in the presence of H_2S but not in the presence of H_2S-activated AzMC or K_2S_n-activated SSP4. The concentration-dependent decrease in SSP4 fluorescence produced by auranofin in HEK293 cells was evident at the initial 4 hr sample and the rate of increase in SSP4 fluorescence thereafter was not appreciably different

between the un-treated and auranofin-treated samples suggesting that the anurafin effects were indirect.

3.4. Effects of combined auranofin and BSO or DEM treatment on H_2S and polysulfide metabolism in HEK293 cells

The combined effects of 10 μM BSO, 100 μM DEM and either 3 or 10 μM auranofin are shown in **Fig. 4**. In these experiments the inhibitors were added to the cells on the first day, whereas the fluorophores were added to different groups of cells on day 1, 2, 3 and 4. This combination of inhibitors nearly completely inhibited H_2S production when applied on the first day and this level of inhibition remained for the duration of the experiment. Polysulfide production was not appreciably affected with 3 μM auranofin when the SSP4 was given on the first day but was decreased with 10 μM auranofin. However, the degree of inhibition of SSP4 fluorescence appeared to progressively wane when the SSP4 was given after day one and by days 2-3 SSP4 fluorescence was greater in auranofin-treated cells. These effects on AzMC fluorescence are consistent with the individual effects of the inhibitors whereas there appears to be cellular over-compensation in polysulfide metabolism. This compensation required auranofin as it was not observed with either BSO or DEM when these experiments were repeated in the absence of auranofin (not shown).

3.5. Effects of conoidin A on H_2S and polysulfide metabolism in HEK293 cells

Conoidin A covalently binds and inhibits peroxiredoxins [60]. The effects of conoidin A on sulfur metabolism in HEK293 cells are shown in **Fig. 5**. Conoidin A concentration-dependently decreased AzMC fluorescence and greatly increased SSP4 fluorescence in both

normoxic and hypoxic HEK293 cells. With 100 μM conoidin A AzMC fluorescence was severely depressed, even at t=0 h suggesting that conoidin A directly interfered with AzMC, and indeed this appeared to be the case. In buffer conoidin A concentration-dependently decreased AzMC fluorescence by itself, in conjunction with H_2S, and after H_2S had reacted with AzMC for 120 min (**Fig. S6**). In all instances there was an apparent threshold of 3 μM (5% inhibition) and at 100 μM more than half of the AzMC fluorescence was inhibited. These results suggest that with conoidin A concentrations above 3 μM the effects on cellular AzMC fluorescence are indirect and probably due to optical quenching. However, the inhibitory effects of 1 μM conoidin A on AzMC fluorescence in cells could be due to direct inhibition of cellular H_2S as this concentration did not affect AzMC fluorescence in buffer. Conoidin A also decreased SSP4 fluorescence in buffer (**Fig. S6**), clearly the opposite of its effects in cells, indicating that conoidin A directly increases intracellular polysulfides.

3.6. Effects of tiopronin on H_2S and polysulfide metabolism in HEK293 cells

The effects of tiopronin, an inhibitor of cystine uptake, on AzMC and SSP4 fluorescence in HEK293are shown in **Fig. 6**. Tiopronin concentration-dependently increased AzMC fluorescence in normoxic HEK293 cells and this was further increased in hypoxic cells. For example, at 21 h 1 mM tiopronin increased AzMC fluorescence 4.9-fold in hypoxic cells compared to a 3.4-fold increase in normoxic cells. After 21 h AzMC fluorescence progressively declined in hypoxic cells but continued to rise, albeit slowly in normoxic cells. In buffer tiopronin increased AzMC fluorescence by approximately 40% (**Fig. S7**) but this was only 1500 to 2000 fluorescence units compared to the increase of over 10,000 fluorescence observed in cells. When added in conjunction with H_2S, tiopronin decreased AzMC fluorescence (**Fig. S7**)

by 40%, but when added 120 min after AzMC and H_2S tiopronin did not inhibit fluorescence between 500 μM and 2 mM and inhibited it by only 25% at 4 mM. These results indicate that topronin substantially increases intracellular H_2S and that this increase is even greater in hypoxic cells.

SSP4 fluorescence was decreased by tiopronin in normoxia and hypoxia with the most notable response in hypoxic cells treated with 1 mM tiopronin. As shown in **Fig. S7**, tiopronin partially inhibited SSP4 fluorescence when added directly to SSP4, when added in conjunction with H_2S and when added 120 min after SSP4 was incubated with K_2S_n. These results indicate that tiopronin likely has little effect on intracellular polysulfides.

3.7. Effects of ebselen on H_2S and polysulfide metabolism in HEK293 cells

Effects of the glutathione peroxidase and peroxiredoxin mimetic on AzMC and SSP4 fluorescence are shown in **Fig. 7**. Ebselen concentration-dependently decreased intracellular AzMC fluorescence in normoxic and hypoxic HEK293 cells but had minimal effects on SSP4 fluorescence with the exception of a delayed increase in fluorescence. The effects of ebselen on AzMC fluoresence in cells are likely indirect. As shown in **Fig. S8**, while ebselen did not directly react with buffer, it decreased AzMC fluorescence by 70% when added concurrently with H_2S but only slightly (<20%) decreased fluorescence when added 120 min after H_2S. Ebselen also profoundly increased SSP4 fluorescence (600%) when added concurrently with H_2S and SSP4 but did not affect SSP4 fluorescence when added directly to SSP4 or 120 min after K_2S_n was added to SSP4. These results suggest that ebselen directly catalyzes polysulfide formation from H_2S. Additional studies are required to clarify ebselen's effects in cells.

277 *3.8. Effects of 2-AAPA on H_2S and polysulfide metabolism in HEK293 cells*

278 The irreversible inhibitor of glutathione reductase (GSHR) and thioredoxin reductase
279 (TrxR), 2-AAPA, concentration-dependently decreased AzMC fluorescence in normoxic and
280 hypoxic HEK293 cells (**Fig. 8**). 2-AAPA also increased cellular SSP4 fluorescence, which at 20
281 μM was 3-4 times that of untreated cells. This increase was noted immediately after application
282 of 2-AAPA and it declined by ~15% within the first 4 h.

283 In buffer, 2-AAPA appeared to directly react with AzMC, increasing fluorescence by
284 approximately 40% at all concentrations (10, 20 and 40 μM; **Fig. S9**). But when 2-AAPA was
285 added to AzMC in the presence of 100 μM H_2S it appeared to slightly decrease fluorescence but
286 this was due to variability in the initial concentrations of fluorophore (t=0 min) and not to 2-
287 AAPA; the ratio of fluorescence between H_2S and 10, 20 or 40 μM 2-AAPA at t=0 min (0.74,
288 1.10 and 0.65, respectively) was not appreciably different from their ratios at 10 min (0.75, 0.99,
289 0.65) or 90 min (0.86, 0.97, 0.70). A minimal (<10%) decrease in fluorescence was also
290 observed when 2-AAPA was added 120 min after H_2S had reacted with AzMC. Collectively,
291 these results suggest that 2-AAPA may directly decrease intracellular H_2S, however this needs to
292 be confirmed.

293 The effects of 2-AAPA on intracellular SSP4 fluorescence, appears to be due to direct
294 interactions between 2-AAPA and SSP4 (**Fig. S9**). Direct addition of 2-AAPA to SSP4
295 produced a concentration- and time-dependent increase in fluorescence that for 10, 20 and 40 μM
296 2-AAPA was 37, 55 and 77 times greater, respectively than SSP4 alone at 90 min. 2-AAPA also
297 increased fluorescence when SSP4 was added to H_2S, albeit to a lesser degree. However, 2-
298 AAPA did not affect fluorescence of SSP4 after the latter had reacted with polysulfides,
299 presumably because at this time there was little un-reacted SSP4 left. Although these results do

not provide any direct information on intracellular polysulfide metabolism, they clearly illustrate the need to carefully evaluate potential interfering reactions with test compounds and fluorophores.

3.9. Effects of aminoadipic acid on H_2S and polysulfide metabolism in HEK293 cells

Aminoadipic acid is a competitive substrate for the system X_c^- transporter and decreases cellular uptake of cystine. Aminoadipic acid did not affect either AzMC or SSP4 fluorescence in HEK293 cells (**Fig. 9**). In buffer aminoadipic acid did not directly react with AzMC or SSP4, but decreased AzMC (15%) and SSP4 (35%) fluorescence when incubated concurrently with H_2S or after 120 min of SSP4 reaction with polysulfides (15%; **Fig. S10**). As there was no cystine in the medium in these experiments, these results suggest that aminoadipic acid has little effect on intracellular sulfur metabolism in HEK293 cells.

3.10. Short-term effects of select inhibitors

A number of the inhibitors used in this study had apparent effects on H_2S and polysulfide metabolism within the first 4 hours. These effects were then examined on a shorter time scale to get a better appreciation of their impact on H_2S and polysulfide metabolism.

As shown in **Fig. 10**, BSO did not appreciably affect AzMC fluorescence, whereas an inhibitory effect of 300 μM DEM was evident within the first hour. This is as expected because BSO inhibits GSH synthesis but does not immediately react with GSH, whereas DEM directly reacts with GSH. By the first hr, 1 mM tiopronin increased H_2S. The effects of 300 μM and 100 μM tiopronin became evident by hrs 2 and 3, respectively. The inhibitory effects of conoidin A on AzMC fluorescence were evident even at the initial (t=0 hr) sample suggesting that these

were due to a direct inhibitory effect on the fluorphore which is supported by the buffer studies (**Fig. S6**).

Conoidin A produced concentration-dependent increases in SSP4 fluorescence within the first 15 min (**Fig. 11**). Although much of the inhibitory effect of 3 μM auranofin appeared to be indirect, as shown by the decrease in fluorescence at t=0 hr, auranofin also appeared to delay the subsequent increase in fluorescence suggesting a possible inhibitory effect in the cells as well (**Fig. 11**).

3.11. PrestoBlue; effects of inhibitors and thiols

The effects of antioxidant pathway inhibitors on cell viability was first examined by incubating HEK293 cells in 5% O_2 with either BSO and DEM (10 and 100 μM, respectively), conoidin A (10 μM) or tiopronin (1 mM) for 46 h in the presence of either AzMC or SSP4 then adding PrestoBlue and measuring PrestoBlue fluorescence one and two hours later (47 and 48h). AzMC and SSP4 fluorescence was monitored at 0, 2, 20, 24, 28 and 44 h to confirm the effects of the inhibitors on H_2S and polysulfide production. The effects of the inhibitors on AzMC and SSP4 fluorescence at 0 and 44 h are shown in **Fig. 12 A,C**, respectively and PrestoBlue fluorescence in cells is shown in **Fig. 12 B,D**, respectively and **Fig. 12 E** shows PrestoBlue fluorescence in PBS. Consistent with previous observations, at 44 h BSO plus DEM and conoidin A decreased AzMC fluorescence, whereas tiopronin increased it. SSP4 fluorescence was slightly increased by tiopronin, greatly increased (~5 times) by conoidin A and decreased by tiopronin.

With the PrestoBlue method cell viability is determined by the reduction of the non-fluorescent resazurin to fluorescent resorufin, which purportedly occurs in the reducing

346 environment of live cells but not in the more oxidizing environment of dead ones [61]. In
347 AzMC-treated cells, Prestoblue fluorescence was greater in the control cells than it was in PBS
348 after 1 h incubation and fluorescence increased further in the cells but not in PBS at hour two.
349 This indicates that the HEK293 cells are still viable after 48 h in 5% O_2. In AzMC-treated cells,
350 both BSO plus DEM and tiopronin produced a further increase in PrestoBlue fluorescence
351 compared to control cells, whereas fluorescence was decreased by conoidin A after 2 h
352 incubation. Prestoblue fluorescence in all SSP4-treated cells was not significantly different from
353 that in PBS.

354 AzMC, SSP4, H_2S (as Na_2S) or mixed polysulfides (as K_2S_n, n=1-7) were then incubated
355 with PrestoBlue in PBS in order to determine they directly affected PrestoBlue fluorescence. As
356 shown in **Fig. S11**, neither AzMC nor SSP4 directly affected PrestoBlue fluorescence, whereas
357 Na_2S doubled PrestoBlue fluorescence by 30 min and K_2S_n immediately increased fluorescence
358 by over 13 fold. Incubation of Na_2S with AzMC or of K_2S_n with SSP4 for one hour prior to
359 addition of Prestoblue eliminated most of the effect of these sulfides on Prestoblue fluorescence.
360 As shown in **Fig. S12A,B**, the effect of K_2S_n on PrestoBlue fluorescence was clearly
361 concentration-dependent and both DTT and 3MP increased PrestoBlue fluorescence, whereas
362 neither cysteine nor cystine appeared to react with PrestoBlue.
363
364 *3.12. Verification of AzMC specificity*
365 The effects of GSH, 3-MP, H_2O_2 and the NO donor sodium nitroprusside on AzMC
366 fluorescence in PBS are shown in **Fig. S13A**. None of these compounds, even at the highest
367 concentrations, had any appreciable effect on AzMC fluorescence compared to H_2S.

AzMC fluorescence increased in HEK293 cells over 48 h and this was slightly, but significantly ($p<0.05$) inhibited by AOA+PPG at 48h. Hypoxia greatly increased AzMC fluorescence and this was inhibited by AOA+PPG at both 24 and 48 h (**Fig. S13B**). These results indicate that AzMC fluorescence reflects cellular H_2S production because inhibitors of H_2S biosynthesis produced the expected decrease in fluorescence.

4. DISCUSSION

Peroxiredoxin, thioredoxin and glutathione/glutaredoxin antioxidant systems reduce H_2O_2 to H_2O by transfering electrons from NADPH via two or more cysteine relay proteins and GSH, the so-called "thiol switches" [21,62-67]. These processes depend on close apposition of the relay molecules and interference with any component will affect their efficacy. This property explains the general observation that the variety of inhibitors used in the present study increase intracellular H_2O_2 and this has been employed in some therapeutic applications, albeit with variable success [22-28,31,59,68-71].

Given the chemical and biological similarities between H_2O_2/H_2O and H_2S_2/H_2S, and the role of cysteine and protein thiols in sulfur metabolism, it seems logical to assume that these antioxidant inhibitors would also uniformly decrease H_2S and increase H_2S_2 if they acted via the canonical ROS antioxidant pathways. Failing to observe this (summarized in **Table 1**), we conclude that these antioxidant systems perform completely different functions with respect to cellular sulfur metabolism and that these may be independent of the ROS-regulating relay switches or they may function in conjunction with heretofore unidentified substrates and enzyme pathways. Our previous observations that catalase, in a NADPH-dependent process, forms H_2S from either thioredoxin, or DTT [55] provides a precedent for this hypothesis.

The 'conventional' and 'unconventional' pathways of H_2S and polysulfide metabolism have been summarized in a recent review 72] and provide a convenient starting point for examining the observed effects of ROS antioxidant inhibitors on cellular sulfur metabolism in the present study. H_2S production from L-cysteine, and to a lesser extent L-homocysteine, via the actions of cystathionine γ lyase (CSE), cystathionine β synthase (CBS), and the tandem activities of cysteine amino acid transferase (CAT) and 3-mercaptopyruvate sulfur transferase (3-MST) have been well established as has been H_2S production from D-cysteine by D-amino acid oxidase and 3-MST. H_2S can also be produced from polysulfides, although this has been less well characterized in cells. Polysulfides are often thought to arise from oxidation of H_2S catalyzed by enzymes such as sulfide quinone oxidoreductase and 3-MST, transition metals, heme proteins and uncatalyzed reactions with nitric oxide (NO). CSE and CBS have also been reported to form cysteine per- and polysulfides ($CysSS_nH$; typically n=1-4) from cystine [73] although this may not be physiologically relevant [74]. Recently, Akaike et al. [74] provided compelling evidence that cysteine is persulfidated and polysulfidated by another, sulfur-donating cysteine in a reaction that is catalyzed by cysteinyl tRNA synthase (CARS). These per- and polysulfide cysteine are co-translationally incorporated into proteins a reaction also catalyzed by CARS. Two CARS, cytoplasmic (CARS1) and mitochondrial (CARS2) are found in eukaryotes with most persulfidation resulting from CARS2, the latter providing the majority of both of mitochondrial as well as cytoplasmic polysulfides. $CysSS_nH$ may account for as much as 70-80% of the protein cysteine and there appears to be a vast interconnected network between polysulfdated proteins and small molecular weight thiols for sulfur signaling, storage and transfer [75,76]. This large polysulfide network is also apparent in our observation of the rapid and substantial

increase in SSP4 fluorescence in the present experiments. Several aspects of the actions of the inhibitors used in the present study deserve special attention in this regard.

A number of studies suggest that Trx and 3-MST appear to occupy a unique position at the junction between H_2S/polysulfide metabolism and antioxidant pathways. As we have shown catalase catalyzed the production of H_2S in the presence of NADPH [55]. It has also been shown that Trx releases H_2S from 3MP-persulfidated 3-MST [77] and both H_2S and polysulfides are released from a 3-MST tri-sulfide that is formed when thiosulfate reacts with 3-MST [78]. Reduced Trx also cleaves the intersubunit Cys-Cys bond of an oxidized, inactivated 3-MST dimer thereby reactivating the enzyme [79]. In the brain, 3-MST produces a variety of cysteine and glutathione per- and polysulfides as well as H_2S and H_2S_n [80,81] and the persulfide concentration has been reported to be nearly equal to the concentration of H_2S [82,83]. Germane to the present study, it has recently been shown that exogenous H_2S prevents H_2O_2-induced cell death in cells treated with auranofin, which was presumed to inhibit Trx. These studies also showed that H_2S didn't affect Trx ptotein levels but favored dissociation of Trx from the endogenous thioredoxin inhibitor, TXNIP (thioredoxin-interacting protein; [84]. See also reviews; [78,85].

4.1. BSO and DEM

Glutathione (GSH) is at the center of cellular redox balance, both as a the most prevalent intracellular ROS buffer and as an intermediary in the GSH/glutaredoxin antioxidant system [62]. Both BSO and DEM decrease intracellular GSH over 24 hrs in primary cortical cultures containing both neurons and astrocytes, however, the mechanisms are different [57]. BSO inhibits γ-glutamylcysteine synthase, the rate-limiting enzyme in GSH synthesis, which increases

436 intracellular cysteine, decreases cystine uptake and increases oxidative stress [57,70]. DEM
437 directly conjugates to GSH which lowers intracellular cysteine and stimulates cystine uptake.
438 DEM may [68] or may not [57] increase oxidative stress.

439 Despite the opposite effects of BSO and DEM on intracellular cysteine, both inhibitors
440 decreased intracellular H_2S without affecting polysulfides. Although we used different cells than
441 those examined by Albano et al. [57], our results suggests that of BSO and DEM have the same
442 effects in HEK293 cells then perhaps as much as half of the H_2S production in HEK293 cells is
443 derived from a sulfur moiety other than cysteine. This is supported by our recent observations
444 that inhibiting CSE, CBS and 3-MST with drugs or by siRNA only decreases cellular H_2S
445 production by half (Olson et al., in review). Evidence that the inhibitory effects of BSO and
446 DEM on H_2S are mediated via GSH is further supported by the relatively rapid inhibitory effect
447 of DEM compared to BSO (**Fig. 10**) which we interpret as the immediate lowering of GSH due
448 to DEM binding compared to BSO which inhibits GSH synthesis but does not directly remove
449 affect GSH.

450 It is possible that H_2S is derived directly from GSH or that GSH mediates some
451 transsulfuration process with another polysulfide than then releases H_2S. The identity of this per-
452 or polysulfide is unknown. However, it is doubtful that this is cystine. Cystine does not appear
453 to be a source of H_2S in HEK293 cells [74] and we showed that it actually decreased intracellular
454 H_2S (**Fig. 2**). Furthermore, the cystine effect cannot be indirect as it slightly increased both
455 AzMC and SSP4 fluorescence in buffer (**Fig S3**). The inability of exogenous cystine to affect
456 BSO- and DEM-induced reduction in intracellular H_2S, despite the fact that DEM increases
457 cystine uptake via induction of system X_c^- transporter [57,86] and BSO decreases it [57] further

458 supports the hypothesis that these compounds do not act through an indirect effect on cellular
459 cysteine or cystine.
460
461 *4.2. Auranofin*
462 Auranofin is an irreversible inhibitor of the seleno-antioxidant enzymes, glutathione
463 reductase and thioredoxin reductase [27,58,59]. Auranofin, like BSO and DEM, increases ROS
464 in cells and in our studies it decreased AzMC fluorescence, as did BSO and DEM (**Fig. 3**).
465 Unlike BSO or DEM, auranofin also decreased SSP4 fluorescence (**Fig. 3**). This latter effect
466 could be attributable to a direct effect on the interaction of SSP4 with polysulfides as auranofin
467 inhibited the reaction of SSP4 with K_2S_n to the same extent (**Fig. S5**), although if SSP4 was
468 administered to HEK293 cells several days after auranofin there was an increase in polysulfides
469 suggesting a rebound from an initial inhibition of polysulfide production (**Fig. 4**). Both AzMC
470 and SSP4 are irreversible RSS fluorophores and provide a history of the amount of RSS
471 produced not the current concentration. By adding SSP4 on consecutive days after auranofin,
472 BSO and DEM, as we did in **Fig. 4**, we were able to follow polysulfide production at different
473 time points and the delayed increase in SSP4 fluorescence in treated cells suggests this rebound
474 effect.
475
476 *4.3. Conoidin A*
477 Conoidin A concentration-dependently decreased cellular H_2S and increased polysulfides
478 (**Figs. 5, 10, 11**) with the effect on the latter appearing fairly rapid, i.e., within the initial few
479 hours and becoming even more pronounced over the ensuing two days. The effects on
480 polysulfides were unlike that produced by any other inhibitor suggesting that conoidin A has

specific effects on polysulfide metabolism. As conoidin A is a covalent inhibitor of peroxiredoxin [60], our results implicate peroxiredoxins in polysulfide metabolism.

Peroxiredoxins are ubiquitously distributed in eukaryotes and well known for their ability to scavenge as much as 90% of intracellular H_2O_2. Recent evidence suggests that they also play key roles in H_2O_2 signaling [64-66]. Like the other antioxidants examined in our study, peroxiredoxins employ cysteine redox switches. As eloquently discussed by Stöcker et al. [66], the conundrum of H_2O_2 signaling is the fact that peroxisomes are far more prevalent in cells and have a much greater affinity for H_2O_2 than do the putative target thiols of regulatory proteins. So the question becomes, how can H_2O_2 selectively react with the appropriate target? Stöcker et al. [66] offer two possibilities, in the first, two-step relay, the H_2O_2-oxidized peroxidase oxidizes an intermediary oxidoreductase which then acts as a relay between the initial peroxidase and target protein. In the second one-step mechanism the peroxidase forms a complex with the target protein and the oxidizing equivalents are transferred directly from the peroxidase to the target. Spatial constraints in the site(s) of oxidant production and effector receptivity can convey additional specificity. Our results suggest a third signaling possibility, H_2S, which is produced in response to a stressor, e.g., hypoxia [87], is oxidized by peroxiredoxins to a polysulfide which then persulfidates the cysteine of the regulatory protein. This mechanism may resolve another conundrum, that being the identity of the oxygen sensor by supporting RSS via H_2S [87] in favor of ROS [88].

4.4. Tiopronin

Glutathione peroxidases (GPx) are a family of selenocysteine-containing enzymes that catalytically degrade H_2O_2 consuming two GSH and in the process generating oxidized

glutathione (GSSG) and H_2O. Tiopronin is a reversible glutathione peroxidase inhibitor believed to exert biological activity in part by ROS scavenging mediated by its thiol group [25,89,90]. Given that GPx consumes GSH it seems likely that tiopronin would increase GSH if GPx was actively metabolizing H_2O_2. We observed that tiopronin produced a profound increase in intracellular H_2S (**Figs. 6, 10**) that could not be attributed to a direct effect of tiopronin on interactions between AzMC, H_2S and tiopronin, all of which tended to decrease AzMC fluorescence (**Fig. S7**). These results support, albeit in a correlational context, the role of GSH in H_2S production; tiporonin increases GSH and H_2S while BSE and DEM decrease GSH and H_2S supporting the concept of H_2S production that is independent of intracellular cysteine.

4.5. Avoiding artifacts; interference, interactions and other considerations

Most analytical methodologies are developed with consideration for specificity regarding analytes with chemical similarities, whereas other potential artifacts are less commonly identified. These pitfalls have been pointed out with respect to ROS [91,92]. We also considered the possibility that fluorophores (and an amperometric H_2O_2 electrode) that were designed to measure ROS also detected RSS and showed that this indeed was the case. Two of these, the redox-sensitive green fluorescent protein, roGFP and the H_2O_2 electrode, were actually far more sensitive to RSS than they were to ROS [41]. Fortunately for the present study, neither AzMC nor SSP4 appear to react with H_2O_2, the NO donor diethylamine NONOate or the free radical dipotassium nitrosodisulfonate (Fremy's salt) and AzMC does not react with polysulfides and SSP4 does not react with H_2S [93]. However, Bibli et al. also reported that AzMC was approximately 4.6, 3.6 and 2.5 times more sensitive to Cys, 3-MP and GSH, respectively than it was to H_2S [93]. We did not observe this dramatic of an effect of Cys, which only increased

AzMC fluorescence by less than 15% (**Fig. S3A**). Nor did we observe any appreciable effect of GSH, 3-MP, H_2O_2 or the NO-donor sodium nitroprusside on AzMC fluorescence (**Fig. S13A**) suggesting that these compounds do not interfere with H_2S-mediated AzMC fluorescence. Furthermore, we also observed that inhibitors of H_2S biosynthesis also inhibited AzMC fluorescence in HEK293 cells indicating that AzMC is an effective probe of cellular H_2S production. While these do not encompass all possible ROS or RSS, we can at least rule out a few of the more probable interfering molecules.

In the present study we looked for three other potential problems, reactions between the fluorophores and inhibitors (left panels in **Figs. S1-S10**), reactions between the inhibitors and RSS (middle panels in **Figs. S1-S10**) and optical quenching or other interference of the RSS-activated fluorophore by the inhibitors (right panels in **Figs. S1-S10**). These experiments showed potential problems with a number of inhibitors that could affect interpretation of their effects on RSS in cells. Conoidin A appeared to interfere with both AzMC and SSP4 fluorescence (**Fig. S6**) which obviated definitive conclusions of its effect on cellular H_2S metabolism but not on polysulfide production as it increased SSP4 fluorescence in cells. Tiopronin also interfered with AzMC and SSP4 fluorescence in buffer (**Fig. S7**) but produced the opposite response in cellular AzMC fluorescence suggesting it too had a direct effect on cellular H_2S production. 2-AAPA appeared to directly react with SSP4 (**Fig. S9**), while ebselen appeared to catalyze H_2S oxidation to polysulfides which resulted in a decrease in AzMC fluorescence and an increase in SSP4 fluorescence (**Fig. S8**). It was not possible to ascertain if either 2-AAPA or ebselen affected cellular RSS. Nor can we conclude that H_2S and polysulfides are the only RSS affected by these inhibitors. Sulfenyl cysteine persulfide (Cys-SSOH) has recently been identified [94] that could escape undetected if not reactive with SSP4 as could

other polysulfoxides [95]. These all need to be resolved as the RSS methodologies are developed.

We have previously shown that a number of metal-centered porphyrins and the porphyrin-containing antioxidant enzyme, catalase, optically interfere with fluorescein-type fluorophores [96] so the effects of the inhibitors in the present study was not entirely unanticipated. We have also shown that a number of the methods used to measure ROS cannot distinguish between ROS and RSS [41]. Most notably, we found that the redox-sensitive green fluorescent protein, roGFP, arguably the gold standard ROS probe [97], is two hundred times more sensitive to RSS than it is to ROS. The redox sensitivity of roGFP is derived from two cysteines inserted into the protein that form an easily reduced disulfide bridge that when oxidized changes the fluorescence absorption spectrum [98]. This clearly explains the responsivity of roGFP to RSS. roGFPs have also been fused with redox catalysts. roGFP fused to peroxiredoxins or Orp1 is reported to provide real time H_2O_2 probes [99-101], and when fused with glutaredoxin it is reported to be a very specific probe for the 2GSH/GSSG redox couple [101]. The sensitivity of all of these modified roGFP probes to RSS will need to be examined before it can be concluded that they are specific for ROS-related events or if they also report RSS activities.

4.6. Is PrestoBlue a polysulfide probe?

Reduction of resazurin (PrestoBlue) by the reducing environment in viable cells produces the fluorescent resorufin and is the basis for this assay [61] and our application in the present stidy (**Fig. 12**). Using this method we showed that HEK293 cells remained viable for up to 48 h in 5% O_2. We also showed that cells treated with BSO plus DEM, conoidin A or tiopronin also

reduced Prestoblue suggesting that these inhibitors also did not affect viability. However, conoidin A halved and tiopronin doubled PrestoBlue fluorescence in AzMC-treated cells, whereas an increase in PrestoBlue fluorescence was not observed in cells treated with SSP4. These findings suggest that either, 1) AzMC and/or SSP4 directly affect PrestoBlue fluorescence, 2) SSP4 kills HEK293 cells, or 3) H_2S and/or polysulfides reduce resazurin to resorufin. Our evidence of these reactions in buffer (**Fig. S11**) suggests the latter. First, neither AzMC nor SSP4 directly affected PrestoBlue fluorescence. Second, if SSP4 killed the cells then we would not expect to see SSP4 fluorescence increase in cells over time nor that this would be differentially sensitive to inhibitors whose effects were shown to be cell-dependent. In the absence of cells, we clearly showed that both H_2S and polysulfides reduce PrestoBlue and that polysulfides are far more efficacious in so doing. **Figure S12** clearly shows that this is not a general effect of thiols as while DTT and 3MP also reduced PrestoBlue, cysteine and cystine did not. This suggests that endogenous H_2S, polysulfides or select thiols also are responsible for reducing PrestoBlue in cells. The greater efficacy of polysulfides compared to H_2S is likely due to the fact that they are better reductants than H_2S [76]. So why does SSP4 prevent any PrestoBlue reduction in cells? We propose that this is due to SSP4 binding to endogenous polysulfides as they are formed before the polysulfides can reduce the Prestoblue. Evidence for this is shown by incubating SSP4 with polysulfides for one hour prior to addition of PrestoBlue which essentially completely inhibits the effect of polysulfides.

Our results also suggested that the mechanism of PrestoBlue reduction in live cells is due, at least in part, to endogenous polysulfides or other thiols. They also suggest the possibility that the supposed oxidation of dead cells which inhibits Prestoblue reduction is not due to increased ROS such as H_2O_2 but due to decreased production of persulfide or other thiol. If both conoidin

A and tiopronin were to increase ROS, as generally assumed (see introduction), then both should have the same effect on PrestoBlue fluorescence, i.e., to decrease it. However, we not only show that these inhibitors have the opposite effects on PrestoBlue fluoresence in cells, but that these effects are identical to their effects on cellular H_2S. Clearly, additional work is necessary to identify the actual thiols that are responsible for PrestoBlue fluorescence.

4.7. Summary

The present experiments suggest that a variety of compounds commonly used to affect cellular redox balance by inhibiting antioxidant pathways also affect cellular H_2S and polysulfides. These experiments also indicate that the effects of these inhibitors on sulfur metabolism cannot be explained by disruption of the canonical ROS antioxidant pathways and subsequent secondary effects of these ROS on sulfur metabolism. Rather they suggest that these inhibitors directly affect cellular sulfur metabolism, potentially via established pathways and/or other mechanisms that remain to be identified.

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Table 1. Summary of effects of ROS inhibitors on H_2S and polysulfides in HEK293 cells.

Inhibitor	Target	H_2S	Polysulfides	Buffer[#]	
				AzMC	SSP4
BSO	γ-GCS	Dec (C-D)	NC	NC	NC
DEM	binds GSH	Dec (C-D)	NC	NC	NC
Auranofin	GSHR	Dec (C-D)	Dec (C-D)	NC	Dec (40)
Conoidin A	Prx	Dec (C-D)*	Inc (C-D)	Dec (60)	Dec (40)
Tiopronin	GPx	Inc (C-D)	NC*	Dec (35)	Dec (80)
2-AAPA	GSHR, TrxR	Dec (C-D)*	Inc*	Dec (30)	Inc (230)
ADA	CSSC uptake	NC	NC	NC	Dec (30)
Ebselen	H_2O_2 scavenger	Dec (C-D)*	Inc*	Dec (70)	Inc (570)

Abbreviations; ADA, aminoadipic acid; AzMC, H_2S fluorophore; BSO, L-buthionine-sulfoximine; C-D, concentration dependent; CSSC, cystine; Dec, decrease; DEM, diethyl maleate; γ-GCS, γ-glutamylcysteine synthase; GSH, glutathione; Gpx, glutathione peroxidase GSHR, glutathione reductase; Grx, glutaredoxin; Inc, increase; NC, no change; Prx, perodiredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase; *, effects may be mediated by

623 direct interaction with fluorophore or sulfides. #, only pronounced and consistent effects on

624 AzMC or SSP4 are considered; (maximum percent change) .

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FIGURE CAPTIONS

Figure 1. Concentration-dependent inhibitory effects of BSO and DEM on AzMC fluorescence in HEK293 cells in either normoxia (21% O_2) or hypoxia (5% O_2) and effects of 10 μM BSO or 100 μM DEM on SSP4 fluorescence in HEK293 cells (right panels). Mean +SE, n=8 wells all experiments.

Figure 2. Effects of 10 μM BSO and 100 μM DEM with or without 200 μM cystine on AzMC and SSP4 fluorescence in normoxic HEK293 cells. Cystine alone decreased AzMC fluorescence but did not significantly affect the greater inhibitory effects of either BSO or DEM on AzMC fluorescence. SSP4 fluorscence was unaffected by cystine, BSO, DEM, alone or in combination. Mean +SE, n=8 wells all experiments.

Figure 3. Auranofin concentration-dependently inhibits AzMC and SSP4 fluorescence in HEK293 cells in both normoxia (21% O_2) or hypoxia (5% O_2). Mean +SE, n=8 wells all experiments.

Figure 4. Effects of 10 μM BSO plus 100 μM DEM and either 3 or 10 μM auranofin on intracellular AzMC and SSP4 fluorescence in HEK293 cells. BSO, DEM and auranofin were added to all wells at day 1 and AzMC added to separate wells at days 1, 2 and 3 and SSP4 added at day 1, 2, 3 and 4. AzMC fluorescence was inhibited throughout the experimental period, whereas SSP4 fluorescence was inhibited at day 1 but by day 2-3 this was reversed and there was more fluorescence in the inhibited cells. Mean +SE, n=8 wells all experiments.

Figure 5. Conoidin A concentration-dependently decreases intracellular AzMC fluorescence and increases SSP4 fluorescence in both normoxic and hypoxic HEK293 cells. Mean +SE, n=8 wells all experiments.

Figure 6. Tiopronin concentration-dependently increases intracellular AzMC fluorescence in normoxic HEK293 cells and this was further increased in hypoxic cells. After 21 h AzMC fluorescence progressively declined in hypoxic cells but continued to rise, albeit slightly in normoxic cells. SSP4 fluorescence was decreased by tiopronin in both environments with the most notable response in hypoxic cells treated with 1 mM. Mean +SE, n=8 wells all experiments.

Figure 7. Ebselen concentration-dependently decreases intracellular AzMC fluorescence in normoxic and hypoxic HEK293 cells but has minimal effects on SSP4 fluorescence with the exception of a delayed increase in fluorescence. Mean +SE, n=8 wells all experiments.

Figure 8. 2-AAPA concentration- and time-dependently decreases intracellular AzMC fluorescence but profoundly, and immediately, increases SSP4 fluorescence in both normoxic and hypoxic HEK293 cells. Mean +SE, n=8 wells all experiments.

Figure 9. Aminoadipic acid does not affect AzMC or SSP4 fluorescence in either normoxic or hypoxic HEK293 cells. Mean +SE, n=8 wells all experiments.

942 **Figure 10**. Short-term effects of BSO, DEM, tiopronin and conoidin A on AzMC fluorescence
943 in HEK293 cells. Mean +SE, n=8 wells all experiments.
944 **Figure 11**. Short-term effects of conoidin A and auranofin on SSP4 fluorescence in HEK293
945 cells. Mean +SE, n=8 wells all experiments.
946
947 **Figure 12**. Effects of BSO plus DEM (10 and 100 μM, respectively), conoidin A (10 μM) and
948 tiopronin (1 mM) on AzMC (**A**) and SSP4 (**C**) fluorescence at 0 and 44 hr and on PrestoBlue
949 fluorescence in AzMC (**B**) and SSP4 (**D**) treated cells 1 h and 2 h after addition of PrestoBlue
950 (hours 47 and 48 after start of experiment). (**E**) PrestoBlue fluorescence after 1 and 2 h in
951 phosphate buffered saline (PBS). Mean +SE, n=8 wells all experiments; *, significantly
952 ($p<0.005$) different from respective control; #, PrestoBlue fluorescence of SSP4-treated cells
953 significantly ($p<0.005$) different from PrestoBlue in PBS; PrestoBlue fluorescence of all AzMC-
954 treated cells was significantly ($p<0.05$) different from PBS.S
955

Supplementary information

Effects of Inhibiting Antioxidant Pathways on Cellular Hydrogen Sulfide and Polysulfide Metabolism

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Running Head: Antioxidant Inhibitors and RSS Metabolism

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977 **Supplementary Figures**

978 **Figures S1-S10.** (**A**, **D**) direct effects of inhibitors on AzMC and SSP4 fluorescence before
979 addition of inhibitors (t = 0 min) and at 10 and 90 min afterward. (**B**, **E**) effects of inhibitors on
980 AzMC and SSP4 in the presence of 100 μM (**B**) or 300 μM (**E**) H_2S. H_2S and inhibitors were
981 added after t = 0 min. (**C**, **F**) effects of inhibitors on fluorescence of AzMC and SSP4
982 preactivated for 120 min with 30 μM H_2S or 30 μM K_2S_2, respectively. Mean +SE, n=4 wells; *,
983 $p<0.05$ compared to AzMC or SSP4 only at same time.
984
985 **Figure S11.** Effects of AzMC, SSP4, Na_2S and K_2S_n on PrestoBlue (PB) fluorescence in
986 phosphate buffered saline. (**A**) BP only, (**B**) plus 25 μM AzMC, (**C**) plus 300 μM H_2S as Na_2S,
987 (**D**) one hr after 300 μM H_2S reaction with 25 μM AzMC, (**E**) plus 10 μM SSP4, (**F**) plus 300
988 μM polysulfide as K_2S_n, (**G**) one hr after 300 μM K_2S_n reaction with 10 μM SSP4. Mean +SE,
989 n=4 wells. Note different y axis scale in **F**.
990
991 **Figure S12**. (**A**) K_2S_n concentration response after 1 h with PrestoBlue; X axis numbers 1, 2, 3,
992 4, 5, 6 correspond to 1, 3, 10, 30, 100, 300 μM K_2S_n, respectively. (**B**) Effects of 300 μM each
993 of cysteine (Cys), cystine (CSSC), dithiothreitol (DTT) and 3-mercaptopyruvate (3MP) on
994 Prestoblue florescence as a function of time. Mean +SE, n=4 wells (buffer) or 8 wells.
995
996 **Figure S13**. (**A**) Effects of potential interfering molecules on AzMC fluorescence in buffer
997 compared to H_2S; glutathione (GSH), 3-mercaptopyruvate (3-MP), peroxide (H_2O_2) and sodium
998 nitroprusside (NP). Mean +SE, n=4 wells. (**B**) Inhibitors of H_2S biosynthesis, AOA+PPG

999 decrease AzMC fluorescence in HEK293 cells in normoxia (21% O2) at 48 h and in hypoxia

1000 (5% O_2) at both 24 and 48h. Mean +SE, 8 wells; *, $p<0.05$.

Fig. 1

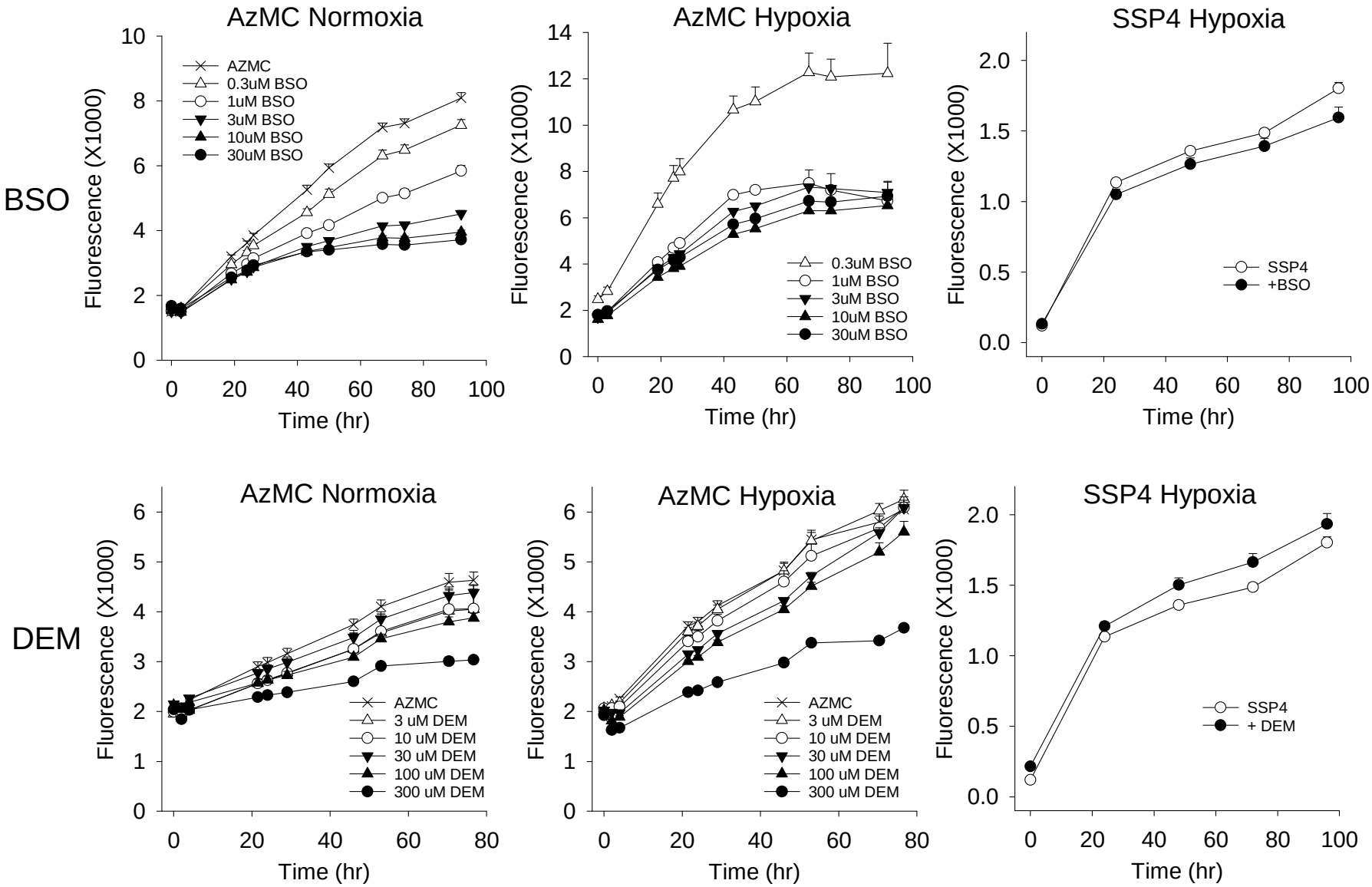
BSO
AzMC Normoxia
AzMC Hypoxia
SSP4 Hypoxia
Fluorescence (X1000)
Time (hr)
AZMC
0.3uM BSO
1uM BSO
3uM BSO
10uM BSO
30uM BSO
SSP4
+BSO
DEM
AzMC Normoxia
AzMC Hypoxia
SSP4 Hypoxia
Fluorescence (X1000)
Time (hr)
AZMC
3 uM DEM
10 uM DEM
30 uM DEM
100 uM DEM
300 uM DEM
SSP4
+ DEM

Fig. 2

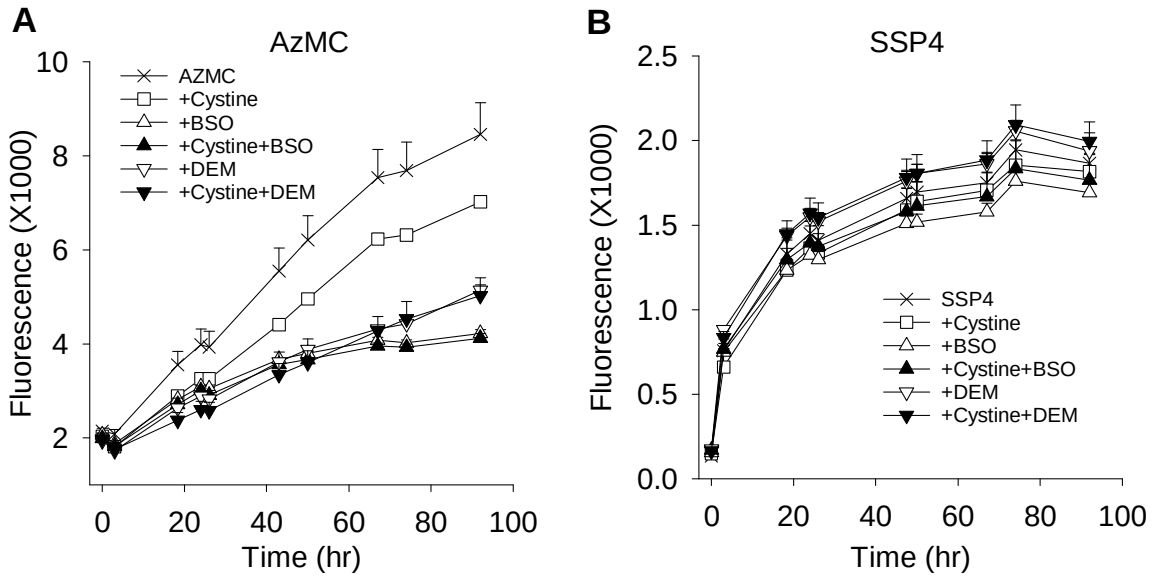
A
AzMC
Fluorescence (X1000)
Time (hr)
AZMC
+Cystine
+BSO
+Cystine+BSO
+DEM
+Cystine+DEM
B
SSP4
Fluorescence (X1000)
Time (hr)
SSP4
+Cystine
+BSO
+Cystine+BSO
+DEM
+Cystine+DEM

Fig. 3

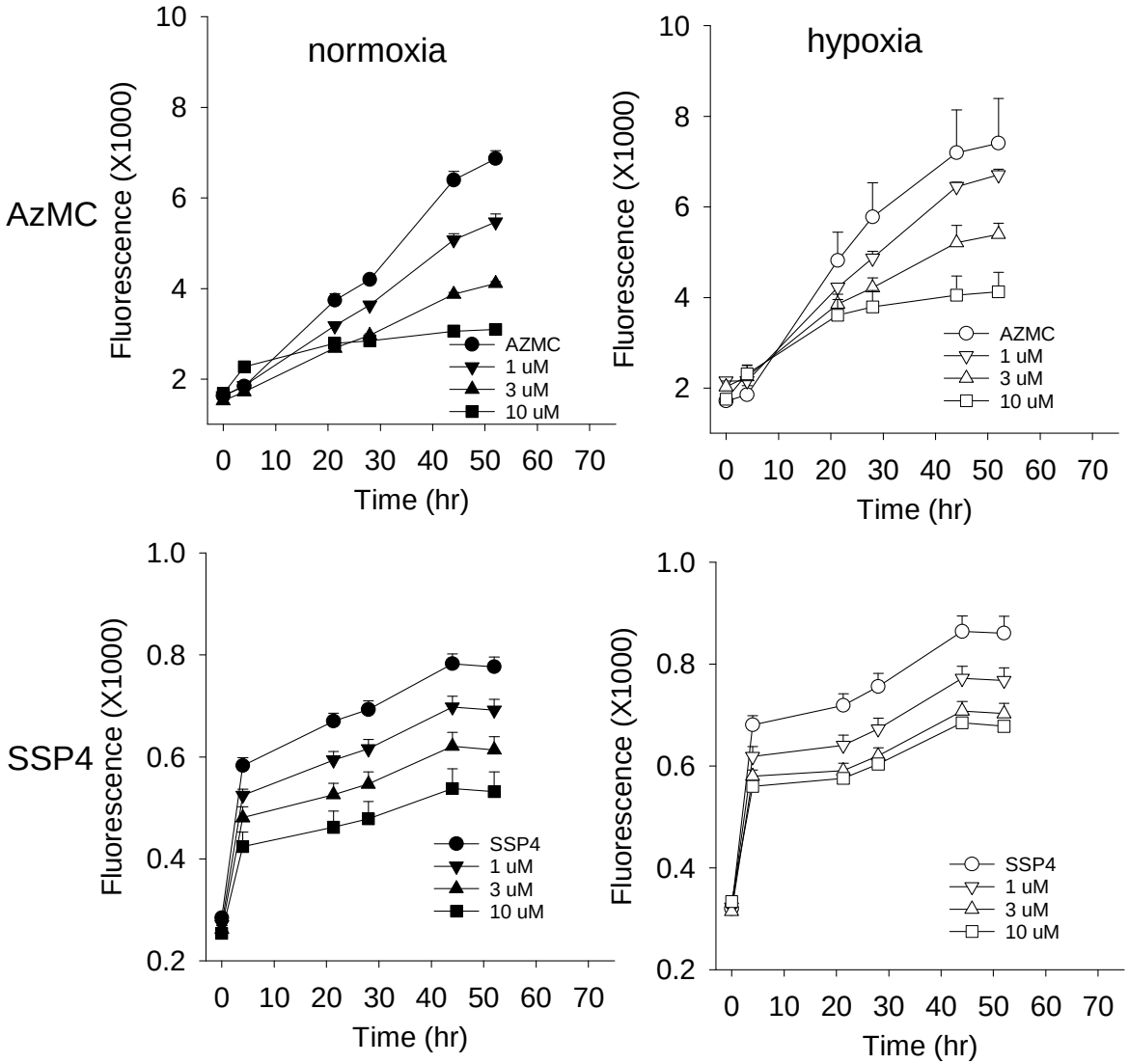
AzMC
SSP4
normoxia
hypoxia
Fluorescence (X1000)
Time (hr)
AZMC
SSP4
1 uM
3 uM
10 uM

Fig. 4

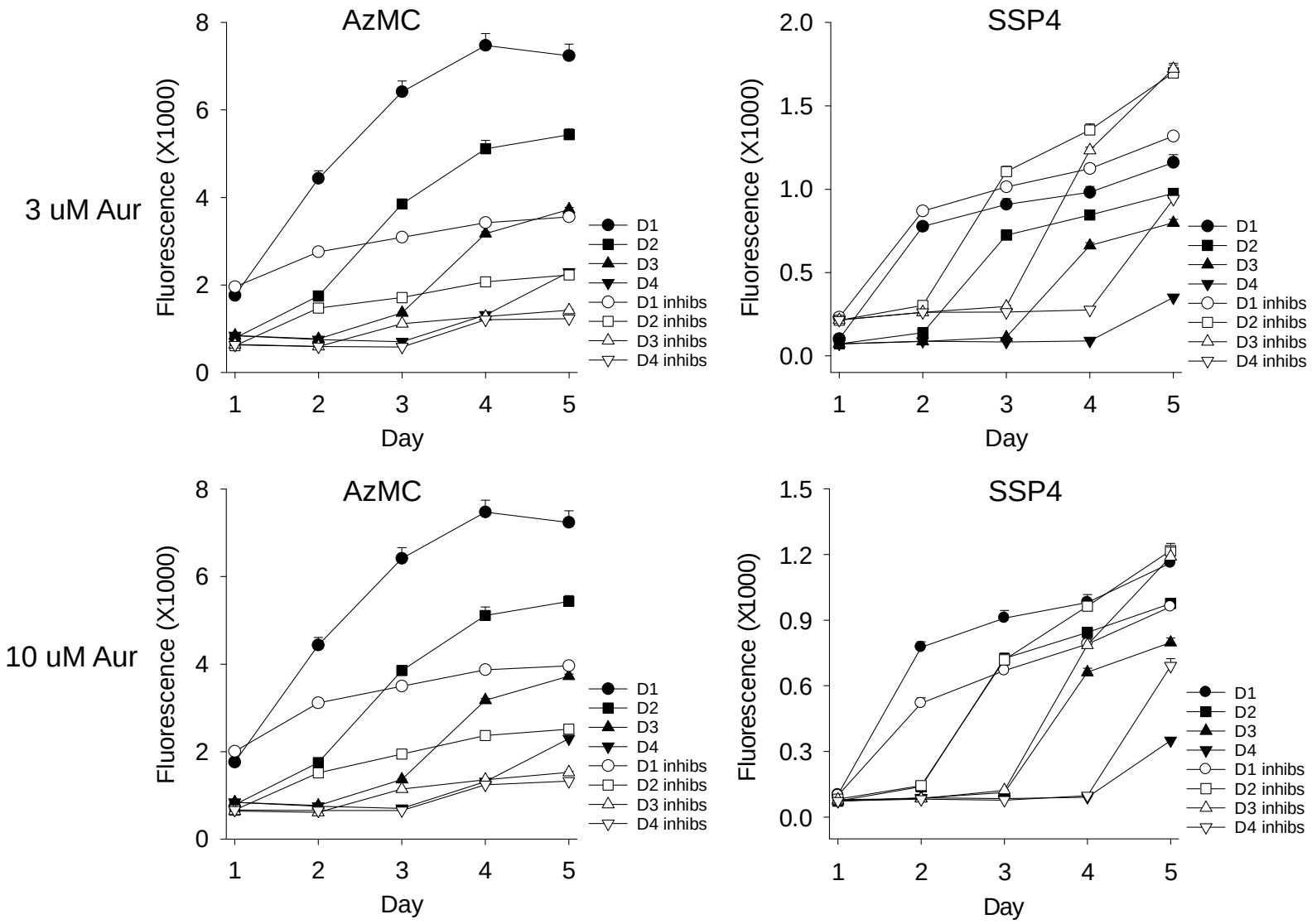
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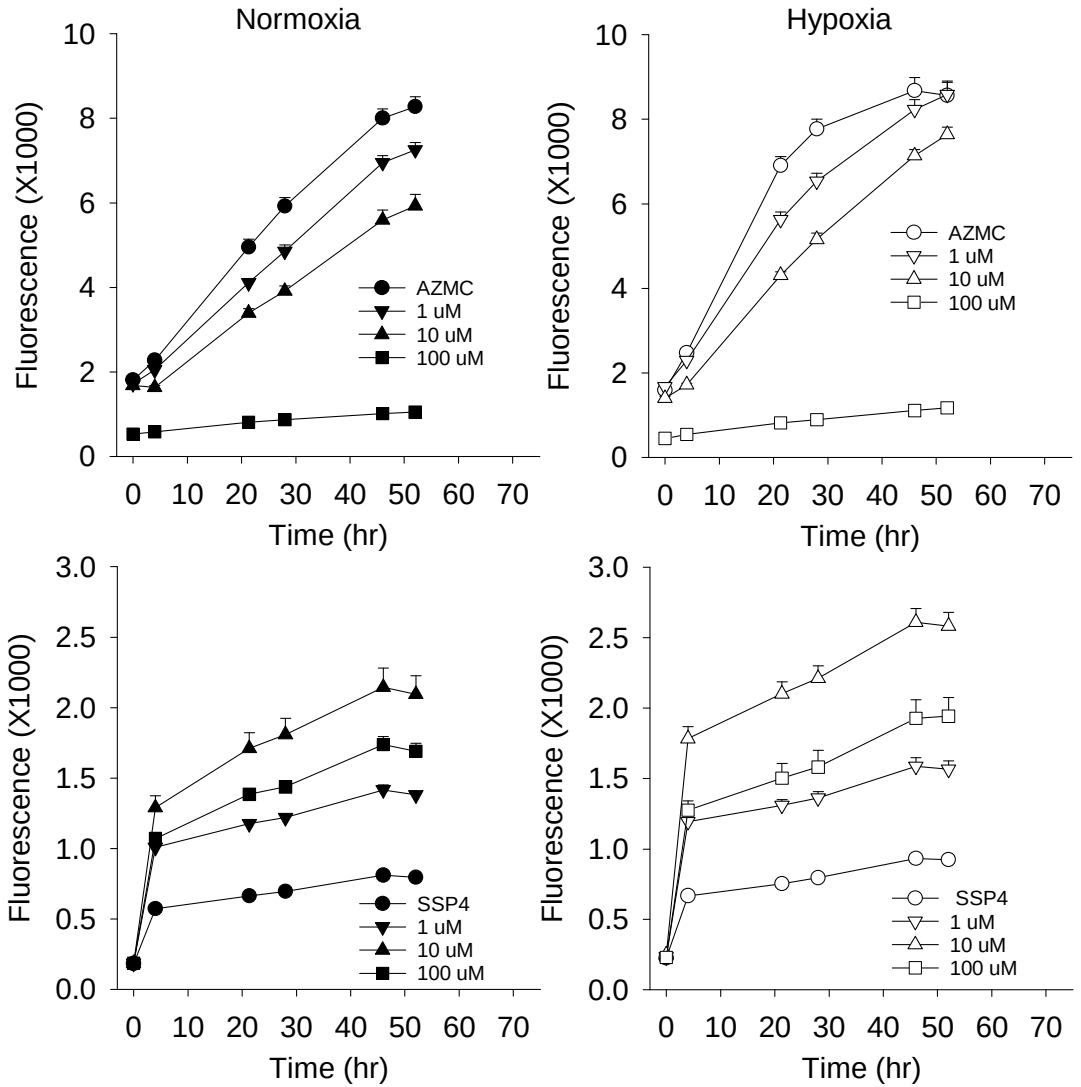
Fig. 5


Normoxia
Hypoxia
Fluorescence (X1000)
Time (hr)
AZMC
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SSP4
1 uM
10 uM
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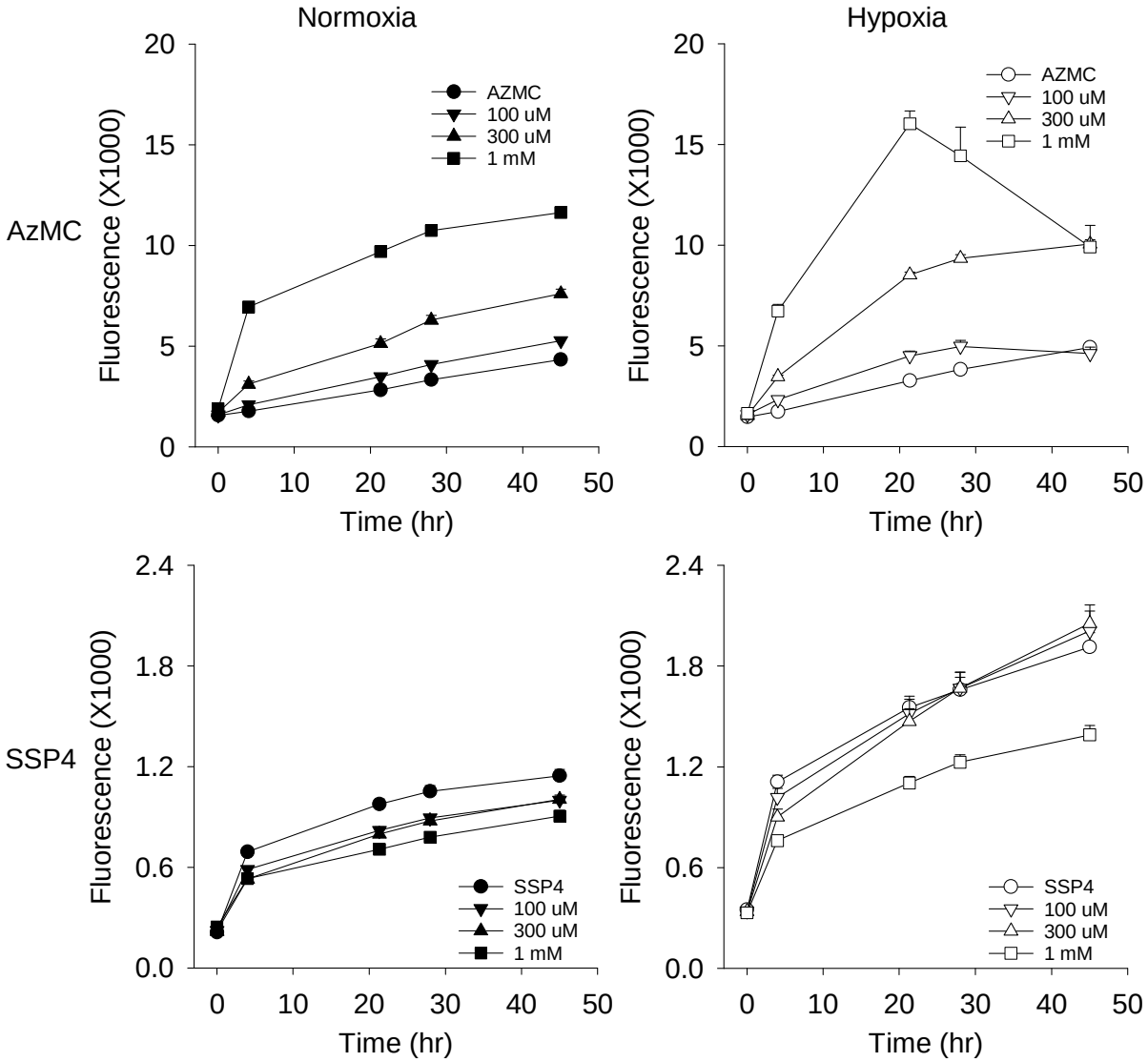
Fig. 6


Normoxia
Hypoxia
AzMC
SSP4
Fluorescence (X1000)
Time (hr)
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100 uM
300 uM
1 mM
SSP4
100 uM
300 uM
1 mM
0
5
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1.2
1.8
2.4
0
10
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40
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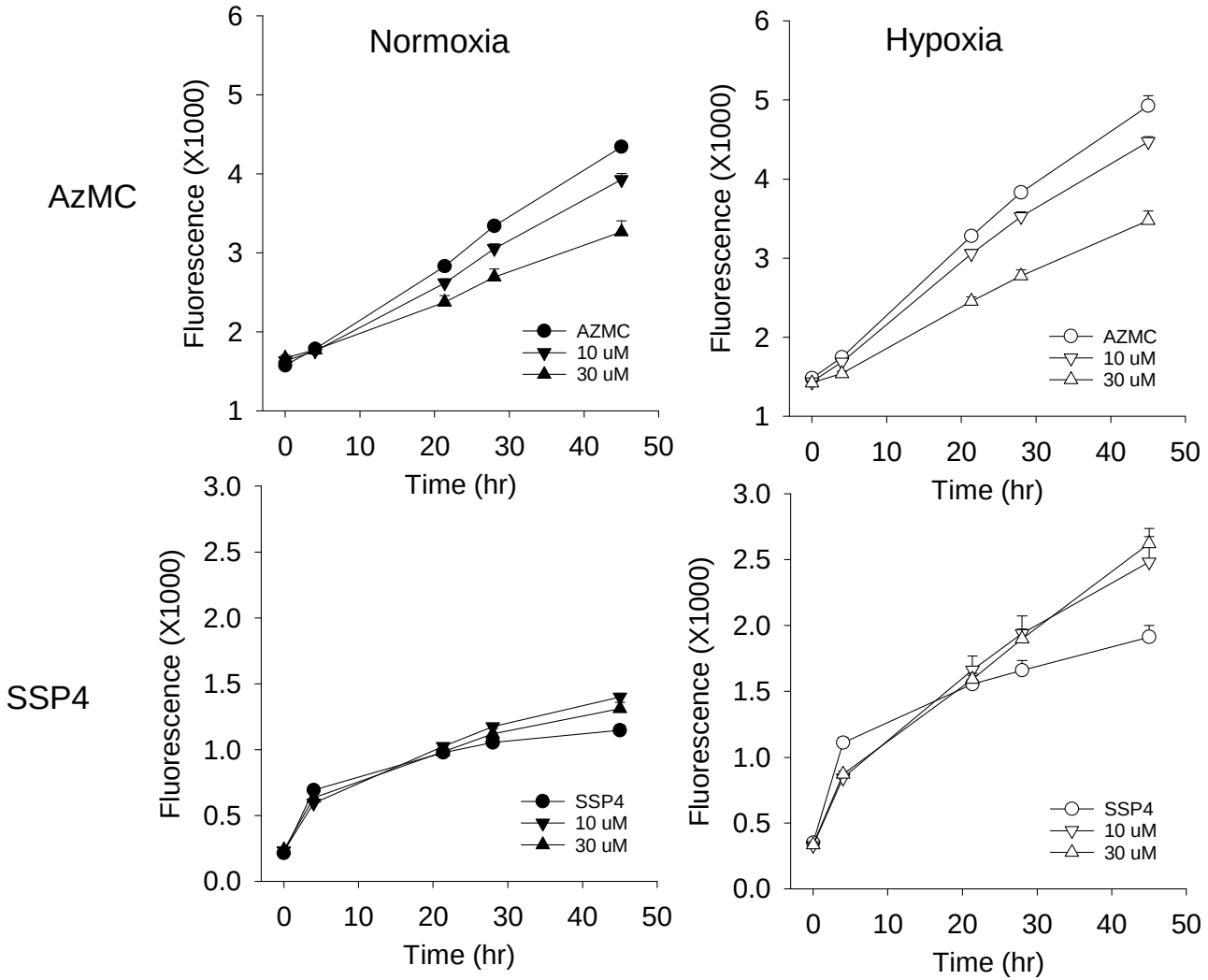
Fig. 7


AzMC
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Normoxia
Hypoxia
Fluorescence (X1000)
Time (hr)
AZMC
10 uM
30 uM
SSP4
10 uM
30 uM

Fig. 8

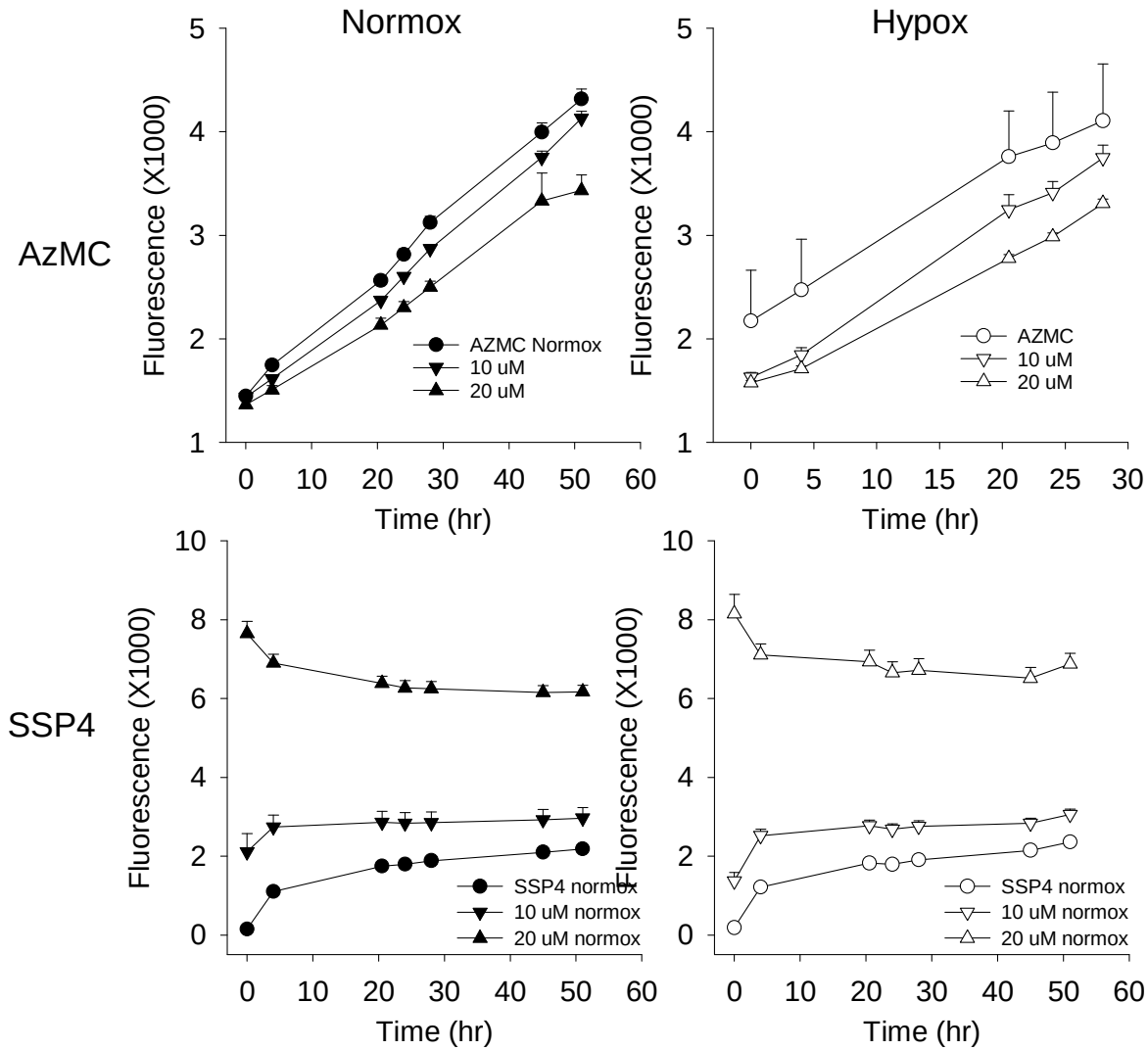

Normox
Hypox
AzMC
SSP4
Fluorescence (X1000)
Time (hr)
AZMC Normox
10 uM
20 uM
AZMC
SSP4 normox
10 uM normox
20 uM normox

Fig. 9

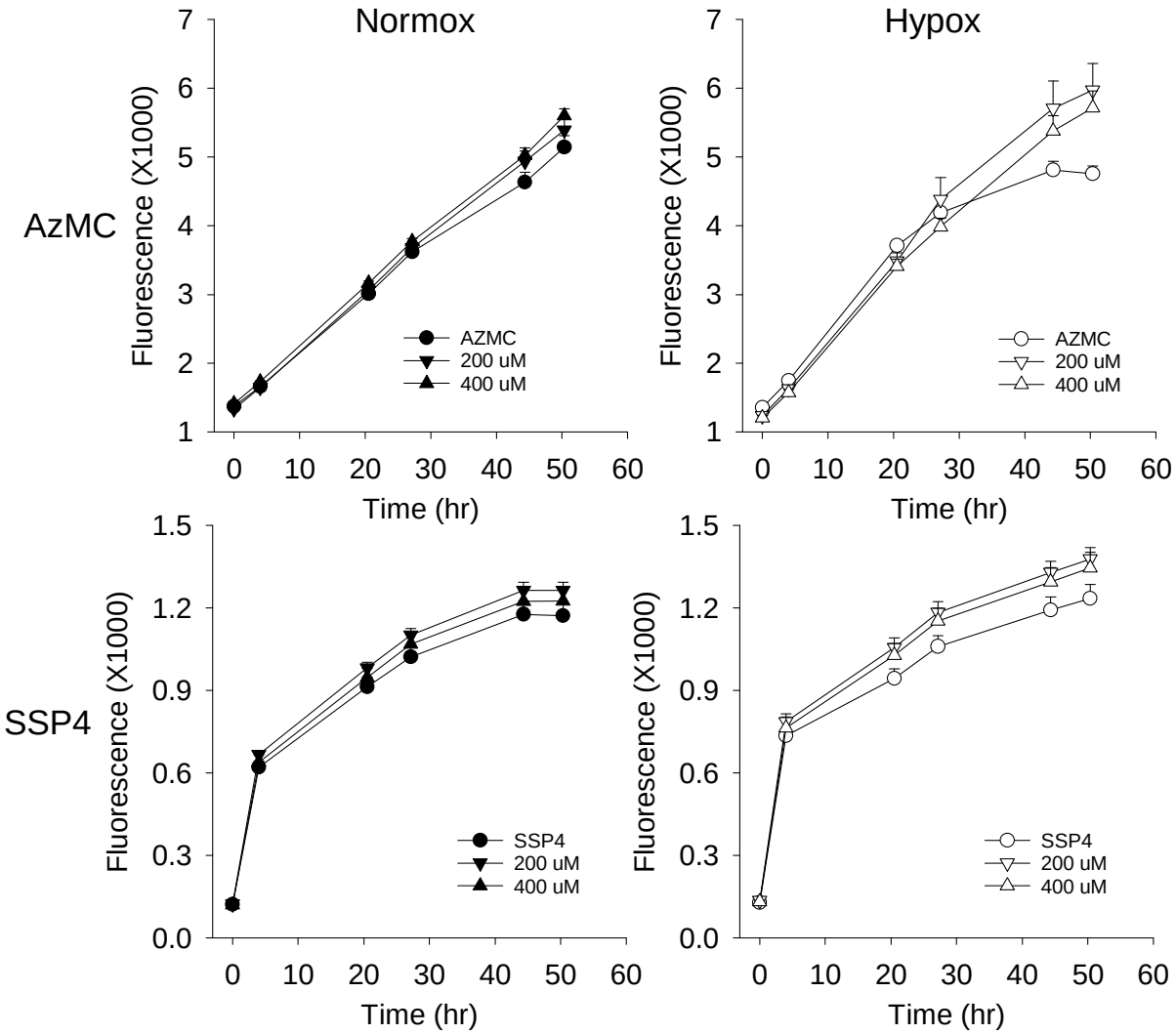

Normox
Hypox
AzMC
SSP4
Fluorescence (X1000)
Time (hr)
AZMC
200 uM
400 uM
SSP4
200 uM
400 uM

Fig. 10

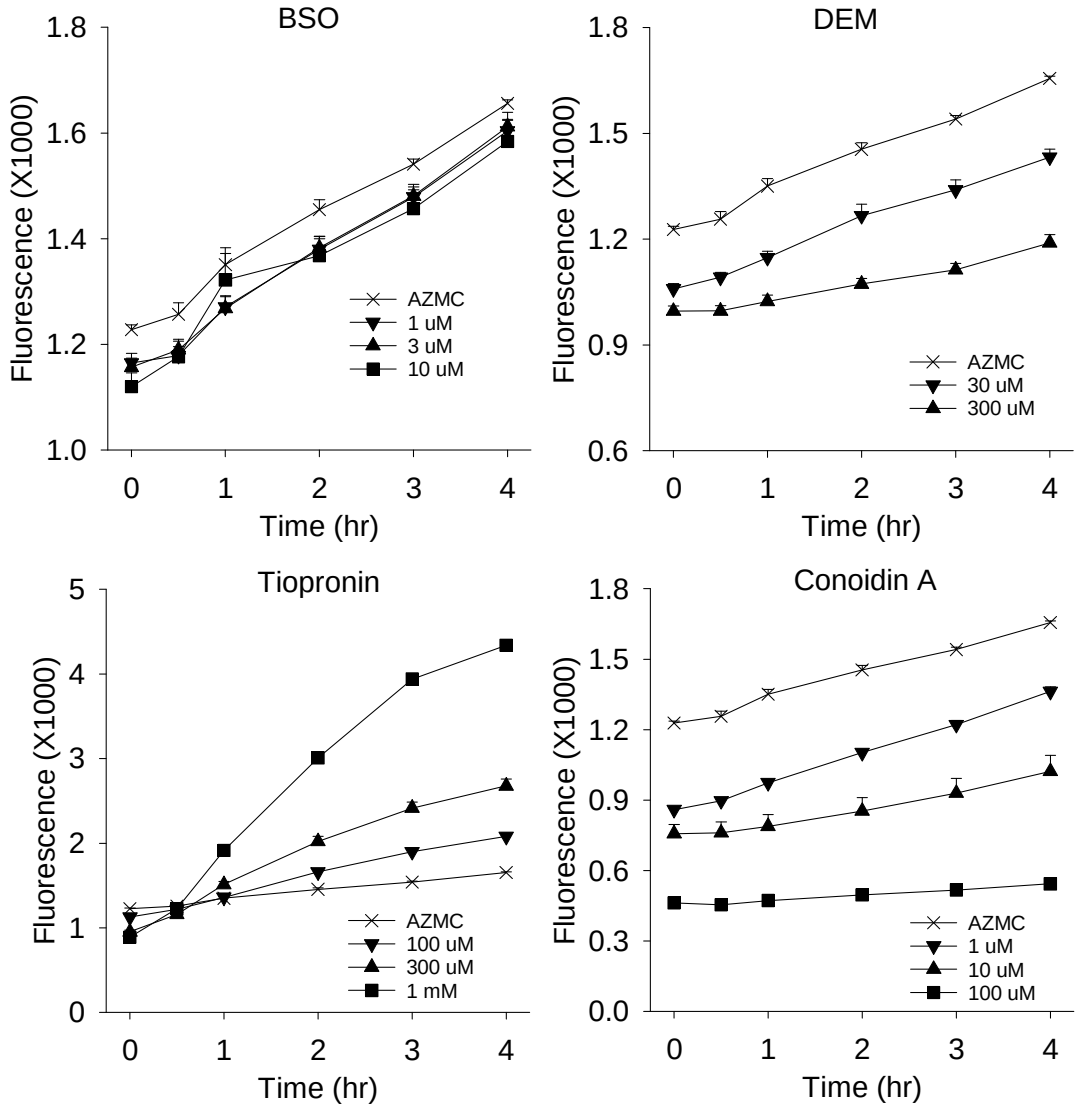

BSO
Fluorescence (X1000)
Time (hr)
AZMC
1 uM
3 uM
10 uM
DEM
Fluorescence (X1000)
Time (hr)
AZMC
30 uM
300 uM
Tiopronin
Fluorescence (X1000)
Time (hr)
AZMC
100 uM
300 uM
1 mM
Conoidin A
Fluorescence (X1000)
Time (hr)
AZMC
1 uM
10 uM
100 uM

Fig. 11

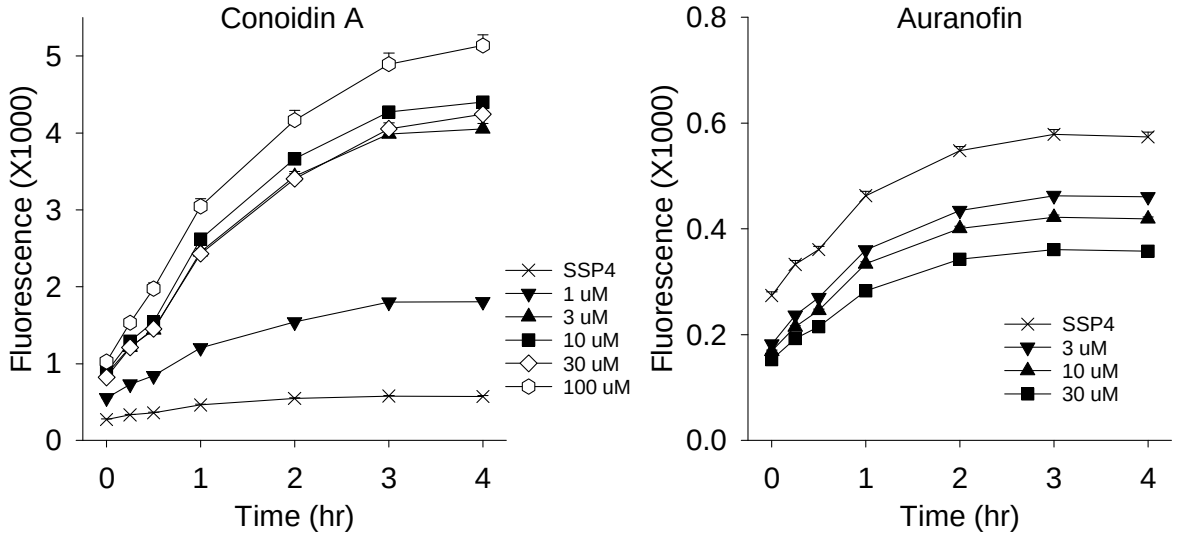
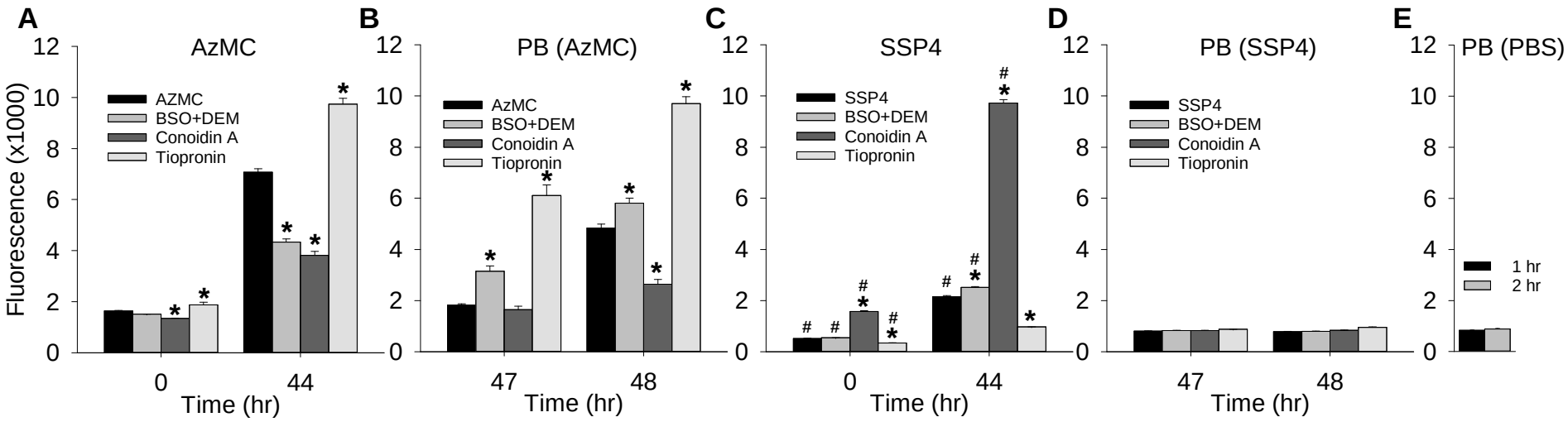

Conoidin A
Fluorescence (X1000)
Time (hr)
SSP4
1 uM
3 uM
10 uM
30 uM
100 uM
Auranofin
Fluorescence (X1000)
Time (hr)
SSP4
3 uM
10 uM
30 uM

Fig. 12


A
AzMC
AZMC
BSO+DEM
Conoidin A
Tiopronin
Fluorescence (x1000)
Time (hr)
B
PB (AzMC)
AzMC
BSO+DEM
Conoidin A
Tiopronin
Time (hr)
C
SSP4
SSP4
BSO+DEM
Conoidin A
Tiopronin
Time (hr)
D
PB (SSP4)
SSP4
BSO+DEM
Conoidin A
Tiopronin
Time (hr)
E
PB (PBS)
1 hr
2 hr

Reactive oxygen species (ROS) and reactive sulfide species (RSS) are chemically and biologically similar.

Evolution of antioxidant pathways is more consistent with RSS metabolism than with ROS metabolism

Here we show that canonical inhibitors of ROS antioxidant pathways affect RSS in HEK293 cells independent of ROS.

These results indicate that antioxidant pathways are involved in RSS metabolism in cells.

RSS may be the actual effector pathway(s) of ROS antioxidant therapies.